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ISOLATION, PURIFICATION, AND CERTAIN PROPERTIES OF THE TYPE B CL. BOTULINUM PROTEINASE

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The proteolytic enzymes of type B Cl. botulinum have been comparatively little investigated. Mashmann offered a general characteristic of proteases produced by clostridia, in particular by Cl. botulinum /1-3/. He established the the presence of two types of proteinases, one of which is activated by certain reducing agents and the other is not susceptible to them. Normal serum inhibited the activity of both types of proteinases, but in a different degree. According to the phases of development of cultures characterized by an accumulation of one or the other proteinase, as well as according to the analogy of the effect of reducing agents upon the intracellular proteases of animals, the first of the above-mentioned clostridia proteinases belong to the category of intracellular and the other -- to that of extracellular enzymes. The peptidades detected in Cl. botulinum cultures also belong to the intracellular enzymes. Millonig isolated from the "Okra" strain cultures a Cl. botulinum B proteinase which proved to be an aminopeptidase, distinguished by a very wide action spectrum /4/. In classifying it among the intracellular proteases, Millonig pointed out that the strain which he had investigated forms only very small amounts of the extracellular proteinase.

The object of the present investigation was the proteinase of a comparatively high proteolytic strain No 175 of Cl. botulinum B., most active at pH levels of 6.0 to 8.0 and apparently related to extracellular proteinases.

Methods

The No 175 strain cultures of Cl. botulinum B were grown on a medium of a hydrolysate of fishbone flour with corn extract /5/. Two modifications of the medium were used, which differed from each other only in the content of soluble calcium salts: 3 to 6 and 15-20 mg percent calculated on Ca^{++}.

As a proteinase substrate we used casein in the majority of experiments, purified by the Shlygin method /6/. The proteinase activity was determined by the Chow-Peticolas method /7/. For a unit of proteolytic activity (PU) in this method the amount of enzyme is accepted which during a period of 15 minutes, at 37°, and a pH of 7.4÷7.8 transforms a definite amount of casein into a state which cannot be precipitated with a 3 percent-trichloroacetic acid. The concentration of casein which has remained unaltered is determined by the degree of absorption of passing light by a suspension formed after the addition of trichloroacetic acid. For this purpose, a series of dilutions of the tested enzymic preparation are mixed with the casein solution (the final concentration of the latter is 0.05 percent), are incubated under the above-indicated conditions and, after the addition of trichloroacetic acid, are compared according to the degree of light absorption with a control mixture, which contains casein in a final concentration of 0.025 percent (half as much as in the tested samples prior to incubation). The tested sample is marked which does not differ, in regard to light absorption, from the control sample, or is very close to it. The multiple of enzyme dilution in such a specimen obviously equals the number of proteolytic activity units per unit of volume of the investigated solution. The variation coefficient of the results of determinations is close to 10 percent.

In studying the effect of a number of factors on proteinase, we evaluated the activity of the latter not only by the Chow and Peticolas method but also by the increase of aminonitrogen. Protein nitrogen was determined as per Kjeldahl, and aminonitrogen -- as per Sorenson.

Investigation results

Proteolytic properties of the cultures

Shown in Figs. 1 and 2 are the proteolytic activity curves in connection with the age of the culture. The greatest activity is reached on the second (in some of the experiments -- on the 3rd day of growth), and it remains at this level until the 5th-7th day, after which it gradually decreases. The concentration of calcium ions in the medium has a considerable effect on proteinase formation; the rise of concentration to 15-20 mg percent (Fig. 1) increases the activity to 80 PU/ml. Addition of glucose (Fig. 2) to 0.25 percent concentration reduces the activity approximately twofold. It should be particularly pointed out that during the

growth periods from 24 hours to days the proteolytic activity of whole cultures did not differ from the activity of the over-precipitate fluid of the cultures which were free of microbial cells.

The data cited in Fig. 3 attest to the fact that reducing agents such as 0.04 M cysteine and 0.002 M $FeSO_4$ not only did not increase but even decreased the proteolytic activity of 48-hour cultures. Normal rabbit-serum only slightly inhibited the activity.

In extracts from washed microbial cells, obtained by the Raynaud method /8/, it was virtually impossible to detect any proteolytic activity (determined, as per Chow). Following a partial lysis of the cells (by 45 percent-90 percent), induced by the addition of gramicidin (20 mkg/ml), or by means of dialysis of two-day old cultures against distilled water, no liberation of proteinase was observed. However, the entry of the culture into the phase of destruction (Fig. 3) was accompanied by the appearance in the culture fluid of proteases activated by cysteine and $FeSO_4$. Similar proteinases were detected in the ultrasonic lysates of cells of the same age.

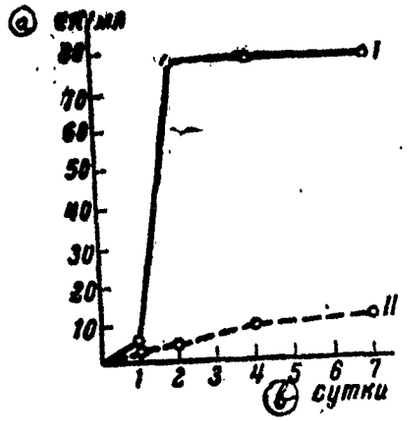

Fig. 1. Proteolytic activity of Cl. botulinum B cultures as a function of the content of calcium ions in the medium (calculated per Ca^{++}):

I -- 15-20 mg percent; II -- 5-6 mg percent.
a -- PU/ml; b -- days

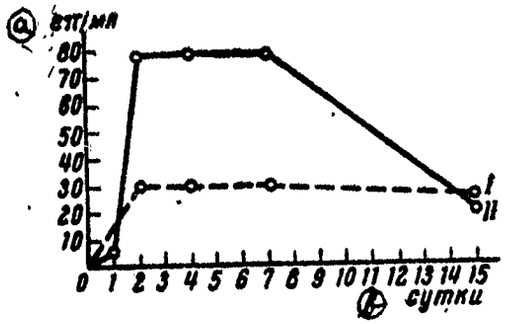

Fig. 2. Proteolytic activity of the Cl. botulinum type B culture during the process of cultivation

I -- with the addition of 0.25 percent glucose;
II -- without the addition of glucose.

a -- PU/ml; b -- days

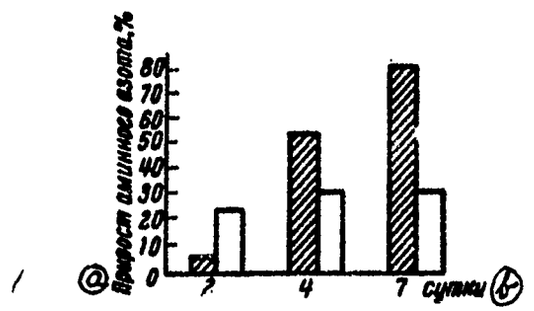

Fig. 3. Effect of cysteine and bivalent iron upon proteinase activity of Cl. botulinum type B cultures of various age.

Black columns -- 0.004 M cysteine and 0.002 M $FeSO_4$ added;
White columns -- no reducing agents added.

a -- increase of aminonitrogen, in percentages
b -- days

Isolation and purification of extracellular proteinase

Reliminary experiments have shown that various precipitators of proteins effective at pH <5.0, and salts of heavy metals irreversibly inactivate proteinase. This circumstance limited the selection of precipitants and the conditions of their use. First, we employed fractionation with ammonium sulfate and ethanol at pH 6.4-7.0. The developed method of proteinase purification is presented in the form of a scheme:

Four-day culture, pH 6.4-6.9

↓

Removal of microbial cells and part of ballast proteins by means of 49 percent saturation with ammonium sulfate (the latter is added in dry form; after dissolving the sulfate, the suspension is kept for 17-19 hours at 6-8° and centrifuged at 500-1000 g for 15-20 minutes at 6-20°).

↓

1st stage Fractionation with ammonium sulfate

Precipitation of proteinase from the over-precipitate fluid at 70 percent saturation with ammonium sulfate (the same precipitation conditions)

↓

Extraction of the precipitate with 1/15 M phosphate buffer solution, pH 7.0, volume -- 1/10 of the initial one.

↓

Removal of the undissolved part of the precipitate by means of centrifugation (500-1000 g, 16-20°, 15-20 minutes). The over-precipitate fluid represents the primarily purified proteinase preparation.

↓

Precipitation of ballast proteins from the primarily purified preparation, at ethanol concentration of 50 percent of

	the volume (the same centrifugation conditions, but the temperature is rom 4° to 12°.
Second stage Fractionation with ethanol at 0°-4°	Precipitation of proteinase from the over-precipitate fluid at ethanol concentration of 75 percent (the same centrifugation conditions).
	Dissolution of the proteinase precipitate in 1/15 M of phosphate buffer mixture, pH 7.0, volume 1/20-1/50 of the initial one. Secondarily purified preparation.
Third stage Additional purification by means of adsorption of admixtures on activated carbon which had been preliminarily washed twice with distilled water	Addition to the secondarily purified proteinase preparation of activated carbon, as per calculation of three grams per 100 ml, at pH 7.0. Vigorous shaking for 10 minutes.
	Removal of carbon by means of centrifugation (10-15 minutes, 16°-20°, 500-1000 g) and filtration through vegetable paper pulp (two to three cm layer).

A comparative description of proteinase preparations at various stages of purification is shown in the table. After a double purification the specific activity varies from 750 to 3410 PU/mg of protein nitrogen. However, the preparations additionally purified with activated carbon possess a fairly constant specific activity which does not depend on the initial culture -- 2860 to 4000 PU/mg of protein nitrogen. In one case (experiment No 5) we succeeded in obtaining after the second purification a preparation with 3410 PU/mg activity of protein nitrogen. However. it is characteristic that this preparation could not be additionally purified with activated carbon. The purified multiple, obtained by means of our method, amounted to 51 to 218, depending on the purity of the initial cultures. The yield in activity of the preparation after the third purification, as a rule, did not exceed 20 percent.

Table

Comparative characteristics of preparations of botulinum proteinase, type B, at various stages of purification (I, II, III)

The specific activity is expressed in PU/mg protein nitrogen

(a) Номер опыта	(b) Удельная активность исходных культур	(c) Удельная активность препаратов			(d) Кратность очистки препаратов			(e) Выход протеиназы в процентах к активности исходной культуры		
		I	II	III	I	II	III	I	II	III
1	17	106	750	3710	6	44	218	29	27	22
2		227	1820	3570	13	101	210	64	33	26
3	48	300	1303	3000	5	27	63	25	23	20
4		363	1445	2860	8	32	60	33	30	24
5	62	217	3410	3570	3	57	58	52	28	13
6		200	1490	3200	3	23	51	56	54	43
7		120	1900	4000	2	30	64	60	57	38

a Number of experiment
b Specific activity of the primary cultures
c Specific activity of preparations
d Purification-multiple of preparations
e Proteinase yield in percentages to the activity of the initial culture

Discussion of results

Strain No 175 of Cl. botulinum B proved to be a very active producent of proteinases upon cultivation on liquid nutritive media of hydrolysates of fish-bone flour with corn extract. The considerable increase of concentration of proteinases in a medium of calcium ions merits attention. There is reason to assume that this effect of calcium ions is not connected with the intensification of the process of formation of proteinases by microbial cells. At present, quite a few data have been accumulated in the literature which permit the assumption that calcium ions considerably increase the stability of a number of bacterial proteinases [9-11]. It is possible that proteinase concentration in the culture depends on the correlation of the rates of formation and destruction of the enzyme. In this case, the retardation of the latter process by means of calcium ions may result in an increase of proteinase concentration.

The reduction of intensity of formation of botulinum proteinase upon addition of glucose is analogous to the effect of certain carbohydrates on the production of proteinase by the cultures of B. pyocyaneous [12]. Although the problem of the functions of bacterial extracellular proteinases is still far from a final solution [12, 13], it is nevertheless natural to assume that in the absence of glucose or any other assimilating carbohydrate as a source of energy, the microorganism is compelled to utilize the protein or products of its incomplete fission in the medium, in which case an increased proteinase formation is essential.

The obtained data attest also to the fact that strain No 175 of Cl. botulinum B is capable of producing at least two proteinases. The first of these is not activated by the reducing agents and is partially inhibited by normal serum. This enzyme reaches considerable concentration as early as during the logrithmic phase of the development of the culture; However, the increase of activity partially continues also during the stationary phase. In the lysates of washed microbial cells no substantial amounts of proteinase could be detected. The second proteinase is elicited only during the phase of the destruction of the culture through its property of being activated by cysteine and $FeSO_4$. In contrast to the first proteinase, it is detected also in lysates of the microbial cells. All this permits us, with sufficient reason, to place the first proteinase in the extracellular category, and the second -- in that of the intracellular enzymes, although we must take into account a certain conditionality of such a division.

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